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13. ABSTRACT *(Maximum 200*

The purpose of this project is to provide a rationale and pre-clinical evaluation of p53-based approaches to growth suppression and therapy sensitization of breast cancer. The project addresses combination approaches employing p53, DNA damaging chemotherapeutic agents, and retinoids. The scope of the second year's study was to continue to evaluate drugs to be used in combination with p53, to correlate DNA repair and DNA damage levels with sensitivity to p53, to identify intermediates in the p53-mediated pathway of apoptosis, and to identify a model system to carry into pre-clinical studies. We have shown that p53 sensitizes several breast cancer lines to doxorubicin (adriamycin), commonly used for breast cancer treatment. We have shown that cell lines with decreased DNA repair and increased genomic instability, are more sensitive to p53-mediated apoptosis, further supporting the rationale for the combined use of p53 and DNA repair inhibitors. We have demonstrated a novel mechanism by which retinoids and possibly other differentiating agents may synergize clinically with cisplatin, providing a rationale for their combined use. Finally, we have begun in vitro evaluation of a promising pre-clinical animal model for metastatic breast cancer which will enable us to evaluate the clinical potential of these p53 combination approaches.

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Ruth A Grieg 7-21-98

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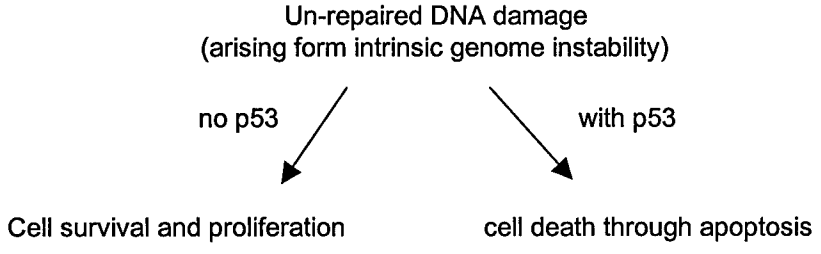
Growth Suppression and Therapy Sensitization of Breast Cancer
DAMD17-96-1-6038
Progress Report #2

INTRODUCTION

This project focuses on the development of biological approaches, primarily p53-based, to growth suppression and therapy sensitization of breast cancer. p53 abnormalities occur in about 40% of breast cancers (1-4), and are a feature of more aggressive tumors showing increased aneuploidy and genetic instability (5,6). Because p53 gene transfer appears to have minimal consequences for normal cells, p53 based approaches to cancer therapy hold promise as effective biological therapies with specificity for tumor cells.

We now know that loss of p53 plays a key role in tumor progression as well as therapy resistance, and this is most likely due to the function of p53 as a modulator of apoptosis following DNA damage (7-11). Karyotypic instability, which occurs in breast cancer and increases with disease progression, generates strand breaks and other forms of DNA damage that can trigger p53-mediated apoptosis. Loss of p53 function would then favor the growth of karyotypically unstable cancer cells by removing the trigger for apoptosis that would eliminate them, as proposed in (12) and as diagrammed below.



This scheme has been supported by studies of mammary tumorigenesis in wnt-1 transgenic mice. These studies showed a correlation between loss of p53 function and increased genomic instability and aneuploidy, suggesting that p53 deficiency relaxes constraints on chromosome number and organization during tumorigenesis. This year we have added further support for this scheme by showing directly that cell lines with decreased DNA repair and increased genomic instability (tasks 2,3) are more sensitive to p53-mediated apoptosis (13, copy attached). Thus the trigger for p53-mediated apoptosis may lie in an abnormal DNA structure generated by the mechanisms that destabilize the genome of these cells.

As a result of losing this apoptotic pathway, tumor cells become more resistant to DNA damaging chemotherapeutic drugs and radiation, which usually kill cells through the induction of apoptosis. We and others have shown that restoration of p53 function in tumor cells that have lost p53, restores apoptosis in response to DNA damaging therapies such as cisplatin and 5-fluorouracil (14,15). Recently we have also shown that restoration of p53 function in T47D

breast cancer cells enhances sensitivity to the DNA damaging drug doxorubicin (adriamycin), a commonly used chemotherapeutic drug for breast cancer. We are presently setting up a metastatic breast tumor model in nude mice that would enable us to test the efficacy of this anticancer drug in combination with p53 gene transfer (see below).

The central hypothesis of this project, supported by our results discussed above (13) is that the level of DNA damage constitutes a key determinant of a tumor cell's susceptibility to p53-mediated apoptosis. Overall our results provide a strong rationale for the combined use of p53 along with DNA damaging chemotherapies for the treatment of breast cancer. We plan to begin testing this approach in animal models in year 3.

BODY OF REPORT

Our objectives for the second year, as outlined in the Statement of Work included (Task 1, continued) conducting *in vitro* drug sensitivity assays in order to identify drugs that worked best in combination with p53, (Tasks 2, 3) correlating DNA repair activity and DNA damage levels with sensitivity to p53, (Task 4) Identifying intermediates for p53-mediated apoptosis, and (Task 6) establishing a tumor model for in vivo studies. We have also used the PCR-stop assay previously set up to study DNA repair to identify a novel mechanism by which retinoids may enhance the benefits of cisplatin therapy.

Results

Drug sensitivity assays. We have tested the response of two p53 mutant breast cancer lines, T47D (estrogen receptor (ER) positive), and MDA-MB-435 (ER negative) to the DNA damaging drug doxorubicin (adriamycin) in the presence and absence of restored wild-type p53. T47D cells were treated with 60 pfu per cell of vector (Ad-p53 or Ad-luciferase) for two hours, and MDA-MB-435 cells were treated with 200 pfu/cell of vector overnight and the next day treated with doxorubicin for 4 hours. Viability was scored at one week post infection (see Materials and Methods). Both cell lines also showed increased sensitivity to doxorubicin following restoration of p53 function (Figures 1 A, B). (Infection efficiencies 60-70%).

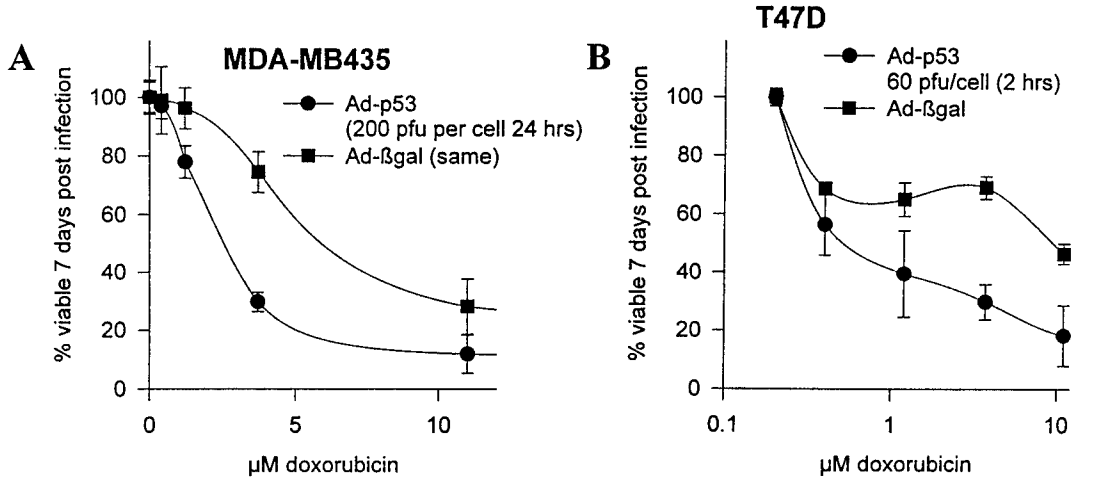
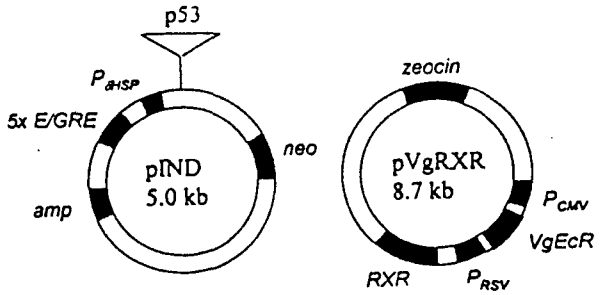


Figure 1. 7 day viability assay of (A) MDA-MB-435 cells and (B) T47D cells following treatment with Ad-p53 or Ad-ß and then doxorubicin for 4 hours.

Selection of T47D clones expressing an inducible p53. In an attempt to obtain clones of breast cancer cells that stably express wild-type p53, we made use of a newly marketed (Invitrogen) ecdysone (muristerone)-inducible mammalian expression system (16). The two vectors pIND (containing the p53 insert) and pVgRXR are shown below and were co-transduced into T47D cells, followed by selection in G418 + zeocin.



two plasmid-ecdysone-inducible expression system.

Previously, using non-inducible systems, we had found it difficult to obtain clones which maintained stable expression of wild-type p53, because of the growth suppressive effect of p53. The inducible system offered a way to circumvent this problem, and in addition appeared to offer significant advantages over previous inducible systems, including the system we initially proposed to use for *in vivo* studies in year 3 (zinc-inducible metallothionein promoter). The ecdysone-inducible system offers extremely low basal expression (which facilitates *in vitro* selection) and high inducibility for both animal studies and *in vitro* cell culture studies. Furthermore, unlike inducible systems based on eukaryotic promoters, there should be no secondary effects in mammalian cells from the insect hormone ecdysone. Thus the behavior of modified tumor cells exposed to ecdysone either in animals or in cell culture can be attributed entirely to the induced expression of p53.

Despite these potential advantages, we did not find that this system facilitated the selection of clones stably modified with wild-type p53. Using the PG13 CAT reporter assay to monitor wild-type p53 expression (see Materials and Methods), we found several clones that induced wild-type p53 following muristerone treatment. As shown in Figure 2, a pool of wild-type p53 expressing clones (p53 pooled clones), as well as one isolated clone (p53 clone 3), were obtained that induced CAT expression from the PG13 CAT plasmid about 3.5 times and 2.5 times, respectively, that of control clones (pIND clone 8 and pIND clone 1).

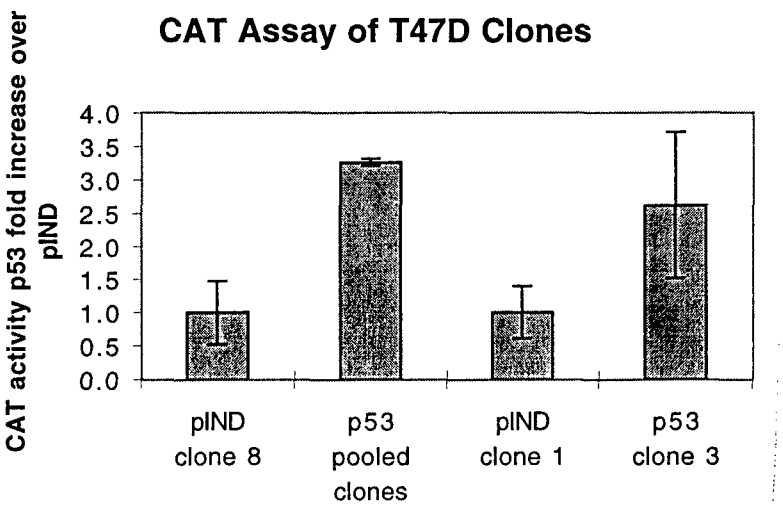


Figure 2. PG13 CAT reporter assays to test for expression of functional wild-type p53 following a 48 hour induction with 1 μM muristerone. in various isolates of T47D cells modified with pVgRXR + pINDp53 (or pIND as control).

When the T47Dp53 pooled clones were induced for wild-type p53 expression by continuous growth in 1 μM muristerone, they were more sensitive to cisplatin, and to the DNA repair inhibitor 3 aminobenzamide (Figure 3 A,B). Overall 6 day growth suppression in 1μM muristerone was about 50%, unlike parental and control vector transduced cells which were not supressed (not shown). The curves shown in Figure 3 do not take into account this additional 50% suppression of viability do to induction of p53 by muristerone, as the data points for each curve represent a percent of the no cisplatin (in A) or no 3-aminobenzamide (in B) point.

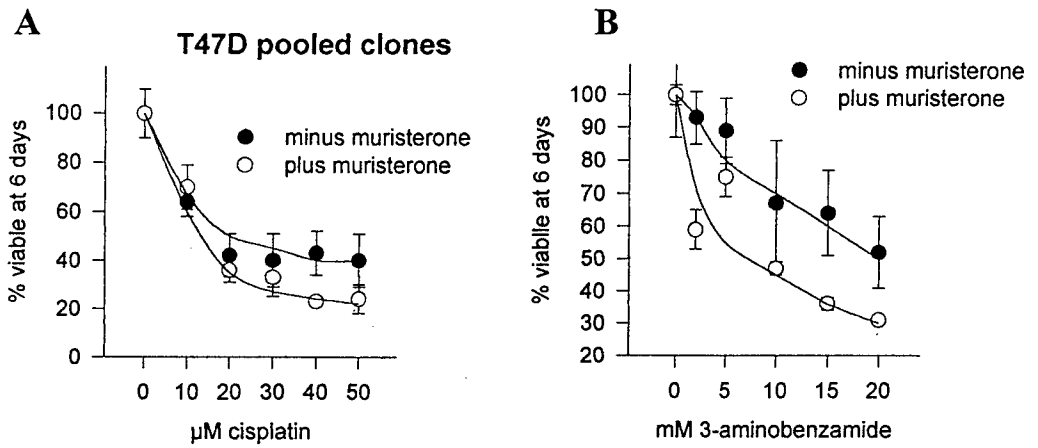


Figure 3. Viability assay of T47Dp53 pooled clones after 6 days of growth in the presence or absence of 1 μM muristerone and following (A) treament with cisplatin (1 hour) on day 1, or (B) continuous treament with 3-aminobenzamide.

These clones will be useful for optimizing p53-mediated apoptosis under conditions of low p53 expression. However, for most studies in which higher levels of expression are needed, we will restore p53 expression using the high efficiency Ad-p53 approach.

Correlation of DNA damage levels and DNA repair inhibition with sensitivity to p53. [This work has been submitted for publication (13) and is summarized here.] In order to obtain direct evidence to support our central hypothesis, namely that the level of DNA damage constitutes a key determinant of a tumor cell's susceptibility to p53-mediated apoptosis, we made use of tumor cell clones that are inhibited in the jun kinase pathway and have a DNA repair defect (17). The jun kinase pathway is induced in response to DNA damage (18) and other forms of stress and leads to the phosphorylation of the c-jun transcription factor (a component of AP-1 and related transcription factors) on positions 63 and 73 in the N-terminal domain.

These jun kinase pathway-inhibited clones (designated T98Gdnjun), were derived from T98G cells by modification with a mutant form of c-jun that acts as a dominant-negative (dn) inhibitor of c-jun N-terminal phosphorylation. The dnjun mutant has amino acid replacements at the two critical jun kinase phosphorylation sites in the N terminal portion (positions 63 and 73) that are phosphorylated by jun kinase in response to DNA damage and various other forms of stress. While the T98Gdnjun clones have growth characteristics similar to the parental T98G cells and

T98G c-jun cells (Table 1), they showed increased sensitivity to the DNA damaging drug cisplatin,consistent with the repair defect we observe (26).

Table 1. *Culture characteristics of T98G clones*

clone	relative doubling time[a]	plating efficiency[b]
T98G	1	47% ± 3%
c-Jun.	1.0 ± 0.14	52% ± 17%
dnJun I-10-10	0.83 ± 0.10	42% ± 13%
dnJun I-10-6	1.2 ± 0.04	45% ± 2%

a) Average of 2 experiments on different occasions, each in triplicate.
b) Average of 3 experiments on different occasions, each in triplicate.

Table 2. *Frequency of methotrexate(MTX)-resistant colonies in T98G cells and in c-Jun and dn-Jun-modified cells.*

Cell were plated at a density of 10^5 cells per 10-cm culture dish and allowed to 4 weeks in the presence of methotrexate at the indicated concentrations. Plates were then stained with 70% methylene blue in methanol and colonies were counted. For analysis of DHFR gene amplification, several colonies were picked at random prior to staining, expanded and cellular DNA was prepared and subjected to PCR analysis

Clone	MTX dose[a]	frequency of colonies per 10^5 cells (average of 3 experiments)	DHFR gene dosage relative to parental
T98G parental	5 x LD50 9 x LD50	2.0 0.3	1
c-Jun	5 x LD50 9 x LD50	2 0	n/t
dnJun I-10-10	5 x LD50 9 x LD50	40 10	3-4
dnJun I-10-6	5 x LD50 9 x LD50	174 16	2

a) LD50 = 0.1 μM (T98G, c-Jun) or 0.04 μM (dnJun I-10-10, dnJun I-10-6).

We have now shown that T98G dnjun cells have markedly elevated levels of dihydrofolate reductase gene amplification, one measure of increased genome instability and intrinsic DNA damage (Table 2). When T98Gdnjun cells are treated with p53 adenovirus, they are more growth suppressed and they undergo apoptosis more readily than do parental cells or control vector-modified cells (Figure 4A,B). This increase in apoptosis (measured by an ELISA assay that detects release of oligonucleosomal fragmetns from the nuclei of cells undergoing apoptotsis)

correlates with a marked cytopathic effect observed 72 hours post infection with p53adenovirus (see reference 13 Figure 2B, copy enclosed). Thus decreased DNA repair and increased genomic instability correlate with increased p53-mediated apoptosis.

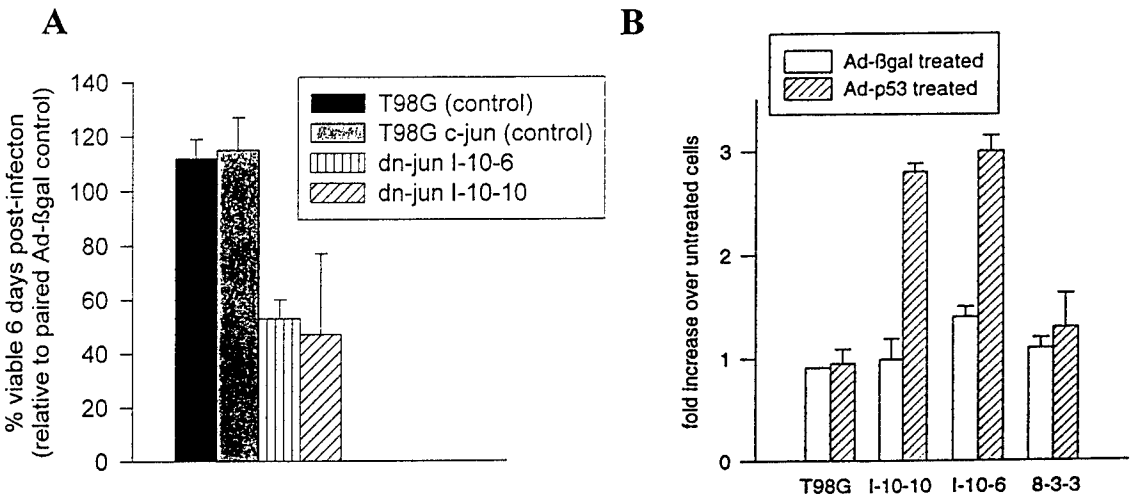


Figure 4. (A) 6 day viability assay of T98G subclones following treatment with Ad-p53, 100 pfu/cell for 3 hours. Viability of each Ad-p53-treated culture is represented as a percentage of the same culture treated under identical conditions with Ad-ßgal (B) ELISA apoptosis assay of cytoplasmic nucleosomes in untreated cells or in cells 48 hours after being treated with Ad-p53 or Ad-ßgal as in (A).

These results could have clinical implication for late stage tumors where DNA repair mechanisms may be upregulated in response to exposure to DNA damaging therapies (19-23). Such tumors may require concurrent down regulation of DNA repair in order to respond optimally to p53 therapy. Down regulation of AP-1 or AP-1 phosphorylation may provide a means to inhibit DNA repair and enhance sensitivity to p53. As shown in the plasmid reactivation assay in Figure 5, T47D cells appear to have a highly efficient nucleotide excision repair (NER) pathway compared to the colon cancer cell line DLD-1. Unlike DLD-1 cells transduced with a cisplatin damaged CAT reporter plasmid where expression is low even 72 hours post transfection, T47D cells are able to completely restore expression of the plasmid by 48 hours, suggesting that DNA repair may be a component of drug resistance in T47D cells. The selection of T47D clones expressing dnjun (below) will allow us to test this possibility.

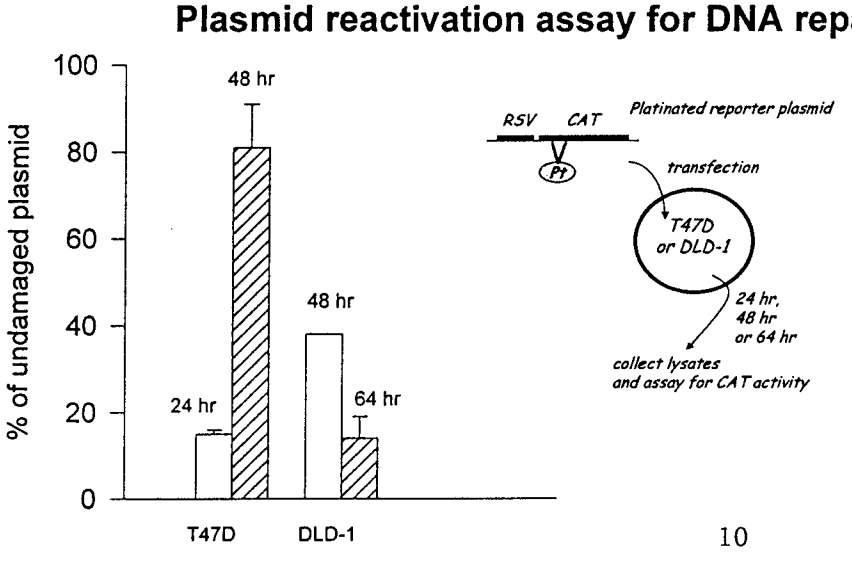


Figure 8. Plasmid reactivation assay showing repair of cisplatin-damaged pRcCAT in T47D cells by 48 post-transfection, and failure of repair in DLD-1 colon cancer cells 64 hours post transfection.

Selection of T47D cells inhibited in the jun kinase pathway. In order to extend these observations to the T47D breast cancer system, we have transduced T47D cells with expression vectors for dnjun or c-jun (control) and have begun selection of clones. As a control, we have also transduced cells with the empty vector (LHCX) used to construct the dnjun vector. These vectors have been described (17).

Demonstration of a novel mechanism by which retinoids may synergize with cisplatin. We have previously been interested in retinoids such as all-trans retinoic acid and 9-cis retinoic acid as possible inhibitors of DNA repair through their suppressive effects on AP-1. While DNA repair may be one target of retinoid action, we have observed a second effect of retinoids that may explain in part their ability to synergize with cisplatin (manuscript in preparation). This second effect could have implications for drug development and p53 mediated apoptosis.

Cisplatin forms primarily intrastrand bifunctional adducts on adjacent guanine-guanine or guanine-adenine dinucleotides, where it induces a 45° bend in the helix toward the major groove (24). The formation of this structural motif may be critical to the anti-tumor activity of cisplatin and explain the differential biological activity of cisplatin compared to its clinically inert trans isomer (transplatin). In particular, cisplatin-DNA adducts are known to suppress transcription to a significantly greater degree than do transplatin-DNA adducts (25). This could be a consequence of the structural features of the transcription complex, where transcription factor-induced distortions in the DNA structure might create preferential targets for cisplatin adduct formation.

We have investigated this possibility by examining cisplatin adduct formation on promoter and downstream regions of the retinoic acid receptor ß gene in T47D breast cancer cells, where this gene undergoes retinoic acid-dependent activation. For controls we have examined regions of two constitutively expressed genes, the dihydrofolate reductase (DHFR) gene, and the hypoxanthine phosphoribosyl transferase (HPRT) gene. Cells pre-treated in the presence or absence of 9-cis retinoic acid for 24 hours and then with 0 mM, 0.5 mM or 1 mM cisplatin for 2 hours were used to compare cisplatin adduct formation on purified genomic DNA. Adduct formation on specific genomic regions (see Table 3 and Figure 5) was then assayed by a quantitative PCR-based assay (PCR-stop assay) under conditions where the inhibition of PCR amplification correlated directly with the extent of platination (see Materials and Methods).

Table 3
Constitutively expressed genes analyzed by PCR-stop assay

Gene	Region amplified	PCR product size
DHFR Dihydrofolate reductase	Exon 1, intron A	272 bases (internal control for PCR)
DHFR Dihydrofolate reductase	Exons 1,2; introns A,B.	1062 bases
HPRTase (hypoxanthine phosphoribosyl transferase)	Introns A - C including exons 1,2	2.7 Kb

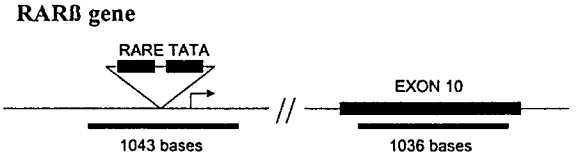


Figure 5. Scheme showing the placement of PCR primers for the RARß gene. Primers were chosen so as to amplify a 1043 base fragment of the promoter region and a 1036 base fragment of exon 10.

PCR primers were chosen so as to amplify regions of about 1000 bases in length and encompassing (1) the RARß promoter, (2) an RAR ß gene downstream region including part of exon 10, and (3) a DHFR gene downstream sequence encompassing exons 1 and 2. A 2.7 kbase region of the HPRT gene encompassing exons 1 and 2 was also amplified and served as a standard to which we could compare levels of platination on smaller fragments. We observed preferential platination of RARß promoter following transcriptional activation compared to downstream regions of the same gene and the DHFR gene, where levels of platination were barely detectable in fragment sizes of 1000 bases. This preferential platination of the RARß promoter was not observed in the absence of transcriptional activation of that gene, suggesting that a structural modulation occurring upon promoter activation may enhance cisplatin binding. Levels of platination per nucleotide were about 3 times greater on the activated RARß promoter than on the HPRT gene. These results are summarized in Figure 6.

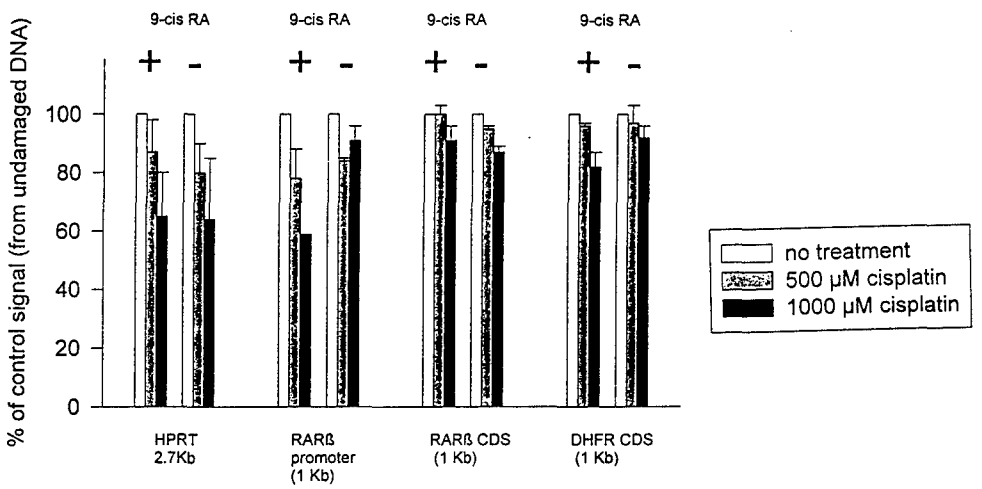


Figure 6. Analysis by PCR stop assay of cisplatin damage on specific regions of T47D genomic DNA following exposure of cells to cisplatin, either in the presence or absence of 1 µM 9-cis retinoic acid. Assays were performed in quadruplicate and results are represented as a percent of PCR product obtained with undamaged template. The relative amplification of the small 270 base fragment of the DHFR gene which is too small a target to sustain detectable levels of damage under any of the conditions used here, served as an internal control for PCR efficiency.

These results suggest that chromatin-remodeling following promoter activation may create sites that are preferentially vulnerable to cisplatin adduct formation. This preference may contribute to the highly effective anti-tumor activity of cisplatin and suggest that drugs that target promoters may have considerable anti-tumor efficacy. Furthermore, retinoids may synergize with drugs such as cisplatin by creating new targets for cisplatin binding in genomic regions where the cell is particularly vulnerable.

Identification of apoptosis intermediates. While loss of apoptosis may occur as a result of loss of p53, it may also occur as a result of disruption of other steps in the pathway. Dysregulation of these other steps may also contribute to tumor progression and drug resistance. We have therefore examined expression of bax, an intermediate in the p53-mediated pathway of apoptosis. Bax-α mRNA expression is high in normal breast tissue, but often very low in breast

tumor samples and cell lines (26). Low bax expression may impede apoptosis and this may result in resistance of tumor cells to chemotherapeutic agents. We have also examined expression of c-myc, as overexpression of c-myc occurs in about 10% of breast cancers (27) and correlates with the onset of resistance to chemotherapeutic agents (28,29). In some systems, c-myc plays a role in apoptosis, suggesting that high levels of c-myc may be tolerated by tumor cells with defects in apoptosis, but not in normal cells. We have performed Western analysis of T47D cells before and after treatment with p53adenovirus and or cisplatin, to determine expression of the bax and myc proteins. Similar analyses of the normal breast epithelial cells line Hs578BST (from ATCC) and the breast cancer lines MDA-MB-435 and MCF7 are in progress.

As shown in Figure 7, expression of bax proetin is low in T47D cells transduced with control virus (Ad-Luc) but is induced following treatment with Ad-p53. Higher expression of bax is observed if Ad-p53 is used in combination with cisplatin (20μM) or 9-cis retinoic acid (2 μM), with the highest levels of expression being achieved with Ad-p53 in combination with cisplatin and 9-cis retinoic acid. Thus the bax component of the apoptotic pathway appears to be intact in T47D cells, and its induction pattern correlates with our earlier growth assays showing maximum growth suppression when p53 was combined with cisplatin and 9-cis retinoic acid. Unlike bax expression, c-myc expression varied little with the different treatments, consistent with other studies showing that c-myc expression is p53-independent.

Figure 7. Western blot showing (A) levels of c-myc and (B) levels of bax in T47D cells following various treatments. Lanes are as follows: Lane 1: Ad-LUC (control), Lane 2: Ad-p53, Lane 3: Ad-p53 + 2 μM 9-cis retinoic acid, Lane 4: Ad-p53 + 20 μM cisplatin, Lane 5: Ad-p53 + 2 μM retinoic acid + 20 μM cisplatin.

Identification of a suitable model system for in vivo studies. We have begun preliminary in vitro testing of the metastatic breast tumor line MDA-MB-435. This model provides the most realistic and rigorous test of the therapeutic approaches we are proposing, as drug resistance becomes a greater obstacle in metstatic disease. The MDA-MB-435 cell line is relatively resistant to adenovirus mediated gene transfer, but high efficiencies of infection (in vitro) can be achieved by increasing the dosage of virus. In other animal tumor models we have used, high levels of adenovirus given in repeated doses are well tolerated and have been effective in combination approaches even with tumor models relatively resistant to Adenovirus, as in the case of the MDA-MB-435 line. P53-Adenovirus is being supplied by Introgen Therapeutics.

Conclusions

We have shown that restoration of wild-type p53 activity sensitizes breast cancer cells to doxorubicin, a common DNA damaging chemotherapeutic drug used in the treatment of breast cancer. These observations extend our earlier observation on p53-mediated sensitization to cisplatin (15), and suggest a broad applicability of p53 as a general sensitizer to DNA damaging therapies. We have also shown that T47D clones modified with an inducible p53, become more sensitive to cisplatin and to the poly-ADP-ribose polymerase inhibitor, 3-aminobenzamide.

Using clonal derivatives of the T98G cell line in which the jun kinase pathway was inhibited, leading to a defect in DNA repair, we have demonstrated a correlation between decreased DNA repair, increased genome instability, and increased sensitivity to p53-mediated apoptosis. This result supports our central hypothesis that DNA damage constitutes a key determinant of a tumor cell's susceptibility to p53-mediated apoptosis, and provides additional rationale for the combined use of p53 with DNA repair inhibitors, as well as DNA damaging drugs. We are presently selecting clones of T47D cells inhibited in the jun kinase pathway in order to extend and confirm the generality of our results with T98G cells. T47D cells display a particularly efficient nucleotide excision repair pathway, and thus represent an ideal model system in which to test the efficacy of a combined therapeutic approach in which drug resistance is reversed by inhibiting DNA repair and restoring p53.

We have addressed the mechanism of synergy between retinoids and cisplatin, as therapeutic synergy between these two agents has been observed in tumor models for breast cancer, and because we have observed the restoration of p53 further improves the therapeutic efficacy of retinoids with cisplatin. Earlier published studies show that retinoid pretreatment enhances the total platinum binding to DNA of target cells. Using the RARß gene as a model, we have observed that the enhanced cisplatin binding occurs preferentially at the promoter region, suggesting that retinods synergize with cisplatin by generating hypersensitive sites to which cisplatin preferentially binds.

We have also begun to address the downstream events in apoptosis that need to occur following restoration of p53 function. Using Western blot assays, we observe in T47D cells that restoration of p53 function leads to enhanced expression of its downstream target gene, bax, and this expression is further induced if cells are also treated with 9-cis retinoic acid and cisplatin. C-myc, an indicator of poor response to therapy, is also expressed in T47D cells, and does not vary with the treatment. These analyses will be extended to other gene products involved in cell cycle regulation, apoptosis, and DNA repair, including p21, p16, $p19^{arf}$, and GADD45 We are also analyzing other breast cancer cell lines and normal breast epithelial cells.

Materials and Methods.

Cell culture. The T47D breast cancer cells used in this work were purchased from ATCC and grown under 5% $C0_2$ in RPMI medium supplemented with 10% fetal calf serum, non-essential amino acids, glutamine, pyruvate and gentamycin.

DNA damage assays based on PCR (PCR-stop assay). We have used a DNA damage assay known as the "PCR stop assay" (30-32) to examine cisplatin adduct formation on different genomic regions. The assay is based on the principle that every DNA lesion, including platinum adducts produced by cisplatin, can potentially block the progression of the Taq polymerase and decrease the yield of a given PCR product. It has been well demonstrated that the degree of inhibition of PCR correlates with the level of platination, indicating that the polymerase is inhibited by every lesion (32). In addition, when whole cells are incubated with varying levels of cisplatin, the degree of inhibition of amplification of a specific PCR fragment from DNA purified from these cells, correlates with platination levels determined by atomic absorption (32), with P (relative PCR efficiency) being related to the average number of adducts, n, per fragment by the Poisson formula: $P = e^{-n}$.

Genomic DNA was prepared from about 10^6 cells treated with 0.5 mM or 1 mM cisplatin either with or without 9-cis retinoic acid, using the Qiagen Qiamp Blood Kit™ following the manufacturer's instructions, and resuspended in sterile H_2O at a concentration of 0.5 mg/ml. PCR reactions are performed in 25 μl containing 50 mM KCL, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 250 μM each of dNTP, 1 μM of the forward and reverse primers and 0.1 μM of the nested primer, 0.25 μl Taq enzyme (Qiagen), and 0.5 μl of Q™ solution (Qiagen). Quantitative amplification is as follows: one cycle (1'30" 94° C), 25 cycles (94°C for 1 minute, 57° C for 1 minute, 70° C for 2'30"), 1 cycle (94° C for 1 minute, 57°C for 1 minute, 70°C for 7 minutes, 4°C to hold). To confirm that the extent of reaction remains directly proportional to amount of template, we have performed control reactions with known amounts of DNA in 2 fold dilutions.

Transfections. For CAT assays we have used a high efficiency transfection method based on cointernalization with an adenovirus capsid. Initially we used capsids to which polylysine tails had been chemically coupled. DNA complexed to the capsid through electrostatic interactions with the polylysine tails then entered the cell via the natural pathway of virus infection as described (33). This approach exploits the endosomolytic and nuclear localization properties of the virus capsid and results generally in higher levels of expression than other approaches. Furthermore, expression begins earlier after transfection than with other approaches, facilitating the plasmid reactivation studies where we want examine expression at early time points. The virus that we used was the replication-defective dl312 virus, purified over CsCl and stored in 10% glycerol in TE at pH 8. We now use a modified approach in which uncoupled virus, DNA, and polylysine are simply mixed together and incubated for one hour with cells (David Curiel, personal communication). We find that this approach, which relies on co-internalization of virus and DNA through fluid phase pinocytosis, is also very efficient, and avoids the need to chemically couple virus to polylysine. Specifically, 6 µg of DNA, 4 µg poly L-lysine, and 10^{10} viral particles in 1 ml of Hepes buffered saline pH8 are added for one hour to cells at 80% confluency in 6 cm plates (about 10^6 cells).

PG13-CAT reporter asasy for wild-type p53 expression. The reporter plasmid PG13 (PG13-CAT) was obtained from Dr. Bert Vogelstein (Johns Hopkins Oncology Center). In this plasmid, the chloramphenicol acetyl transferase (CAT) gene is under the control of a wild-type p53-specific binding site (34). Cells to be assayed for wild-type p53 expression were transfected

(see above) with PG13 and incubated for two days at 37 oC in 5% CO2. Cell lysates were prepared and assayed for their ability to acetylate 14C-chloramphenicol as revealed by thin-layer chromatography of the reaction products (34). Alternatively, triplicate transfections with PG13 were performed in 24 well plates and lysates were assayed for their ability to acetylate chloramphenicol using 3H-Acetyl CoA; in this case, the reaction products were extracted and quantitated by scintillation counting (35).

96-well viability assay. Cells are plated at 1000 cells per well in 96-well plates and treated the next day with various doses of drug. Quadruplicate wells are used for each condition. 5-6 days later (during which time control wells are in exponential growth) viability is measured by the MTT assay. This assay measures the bioconversion of a non-colored tetrazolium compound, MTT into the colored product, formazan. OD 490 nm is then measured using an ELISA reader.

Western blots. Cell lysates were adjusted to a protein concentration of 5 mg/ml and about 50 μg were electrophoresed on a 15% acrylamide gel, transferred to an 0.45 μm PVDF-plus transfer membrane (Micron Separations, Inc.) and probed with rabbit polyclonal anti-bax or mouse monoclonal anti-c-myc (Santa Cruz Biotechnology, Inc.) at 1 μg/ml followed by HRP-secondary antibody (1:1000) using the protocol supplied by the manufacturer. The blot was then treated with ECL Detection Reagents (Amersham) and exposed to Kodak Biomax MR film for 2 minutes.

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GENOMIC INSTABILITY AND SENSITIVITY TO P53-MEDIATED APOPTOSIS IS MODULATED BY THE JNK PATHWAY.[1,2]

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ABSTRACT

This study examined how the Jun kinase stress-activated pathway affects tumor cell growth and genome stability, and how it modulates cellular responses to wild-type p53 expression. N-terminal phosphorylation of c-Jun by Jun kinase is an early response to DNA damage and may also play a role in cellular transformation. Inhibition of that response may render a cell less able to respond to DNA damage. We have previously shown that T98G glioblastoma cells, stably modified to express a dominant negative inhibitor of c-Jun phosphorylation (T98GdnJun) have a DNA repair defect, and are more sensitive to cisplatin and UV-induced DNA damage. Here we show that T98G-dnJun cells have growth characteristics similar to unmodified parental T98G cells, as well as T98G cells modified with c-Jun. However, T98G-dnJun cells have elevated levels dihydrofolate reductase gene amplification, one measure of increased genome instability. Furthermore, when T98G-dnJun cells, which express an endogenous mutant p53, are treated with wild-type p53 adenovirus, they undergo apoptosis more readily than do parental T98G cells or c-Jun-modified cells. Thus we show that the Jun kinase pathway plays a role in the maintenance of genome stability, and that the increased genome instability arising in T98G-dnJun cells correlates with an increased sensitivity to p53-mediated apoptosis. The results may have clinical implications for late stage tumors, where upregulation of cellular stress response pathways and DNA repair may contribute to survival in the face of increasing genome instability or following exposure to DNA damaging therapies. Optimal response to p53-based therapies may require concurrent down-regulation of these pathways.

INTRODUCTION

Tumor progression involves the rapid accumulation of genetic alterations that promote cell proliferation and enable cell survival in changing environments. Genomic instability, one of the unique features of cancer, may provide the driving force for progression by facilitating these rapid genetic alterations (see 1). Nevertheless, this instability generates strand breaks and other forms of DNA damage that serve as triggers for cell elimination. There is therefore selective pressure on the cell to modulate its stress response and apoptotic pathways so as to insure survival while accommodating this increased genetic instability.

One important component of the cellular stress response in the presence of increasing genome instability is loss of the tumor suppressor p53. p53 modulates apoptosis in response to DNA damage, most likely as a result of its ability to recognize and bind to damaged sites in DNA, including single stranded ends (2) and abnormal structures known as insertion-deletion loops (3). Numerous studies link loss of p53 with increased genome instability (4-7), aneuploidy (8,9), and tumor progression (10).

Another component of the adaptive stress response to DNA damage is the Jun kinase/Stress-activated protein kinase pathway (JNK/SAPK) (11). Jun kinase phosphorylates the c-Jun component of the AP-1 transcription factor on serines 63 and 73 in the N-terminal domain. This phosphorylation activates transcriptional transactivation by AP-1 and is induced in response to DNA damaging treatments such as UV irradiation

(12), cisplatin (13,14), etoposide (15), camptothecin (13). Phosphorylation of c-Jun is induced in response to oncogene expression and has been suggested to be required for c-Jun plus Ha-ras co-transformation of rat embryo fibroblasts (16,17).

Recently we have observed that glioblastoma cells inhibited in c-Jun phosphorylation are unable to repair cisplatin adducts in genomic DNA, and are significantly more sensitive to DNA damaging treatments such as cisplatin and UV irradiation (14), but not to treatments that inhibit microtubules, such as docetaxel (Lebedeva and Gjerset, unpublished). Thus the Jun kinase mediated stress response is likely to include induction of DNA repair, possibly through transcriptional transactivation of AP-1 regulated genes such as topoisomerase I, and DNA polymerase ß. In this way the JNK stress response pathway might provide an additional mechanism by which genomically unstable cancer cells achieve enhanced survival.

In many tumor types, restoration of p53 function is often sufficient to insure restoration of apoptosis. In cases where p53 alone fails to trigger apoptosis, we have observed a synergistic effect p53 and DNA damaging treatments (18,19). In this study we have investigated the potential synergistic effect on tumor cell growth achieved by restoring p53 and simultaneously inhibiting the JNK pathway. Inhibition of the JNK/SAPK pathway was achieved by modifying T98G glioblastoma cells with a dominant negative inhibitor of c-Jun phosphorylation with serine to alanine replacements at codons 63 and 73 as previously described (14). We observe increased genomic instability in tumor cells modified in this way, and enhanced sensitivity to p53.

MATERIALS AND METHODS

Cell Lines. T98G glioblastoma cells were obtained from Dr. Hoi U (University of California, San Diego) and cultured at 37° C in 10% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% newborn calf serum. The T98G clones modified to express mutant Jun, termed T98G-dnJun-I-10-10 and I-10-6 (see 14), were cultured in the same way as were T98G cells except that hygromycin was added to the culture medium to 100 µg/ml. T98G-dnJun is stably modified to express a dominant negative mutant of c-Jun (the mutant was obtained by site-directed mutagenesis by M. Karin and colleagues (17)). The dn Jun protein has serine to alanine substitutions at positions 63 and 73, two sites of DNA damage-induced phosphorylation, and can therefore not be phosphorylated at these sites. Control T98G cells modified with a c-Jun expression vector were cultured similarly and in the presence of 100 µg/ml G418.

Virus. Replication-defective Adenoviruses (Ad-p53 and Ad-ßgal) in which the human p53 coding sequence or the bacterial ß-galactosidase gene, respectively, replaced the viral early region E1A and E1B genes were provided by Introgen Therapeutics, Inc. (Houston, TX).

Infection conditions. Cells at 80% confluence were placed in DMEM supplemented with 2% heat-inactivated fetal bovine serum and infected for 3 hours at a multiplicity of 100 pfu per cell. The efficiency of infection was determined by X-gal staining a sample of the ß-gal virus-infected cells (see 18), and was usually ≥ 50%.

Viability and growth assays. Following infection, triplicate aliquots of cells were replated in 96-well plates at a density of 1000 cells per well. Plates were incubated for 5-7 days and surviving cells were determined by adding 50 µl of a solution containing MTS (3-(4,5'-dimethylthiazol-2-y1)-5-(3-carboxymethoxylphenyl-2-(4-sulfophenyl)-2H-tetrazolium inner salt) and PMS (phenazine methosulfate) (both purchased from Promega, Madison, WI.) for 1 hour and determining A590 nm of the resulting formazan product, following procedures provided by the manufacturer. For growth assays, cells were plated at 1000 cells per well in 96-well plates. On successive days from day 1 through day 8, triplicate samples were stained with MTS as described above.

Analysis of dihydrofolate reductase gene amplification frequency. LD50 values for methotrexate were determined for the cell lines to be tested. Cells were seeded at a starting density of 10^3 cells per cm^2 and allowed to attach for 16 hours. Methotrexate (Sigma, St. Louis, MO) was then added to a concentration of 5 x LD50 or 9 x LD50. Medium with fresh methotrexate was replaced weekly. When colonies developed and reached a size of about 100-200 cells (about 5 weeks), plates were washed in PBS and stained with 1% methylene blue in 70% methanol.

Analysis of gene copy number. Following selection in methotrexate as described above, several clones were picked at random and expanded. Genomic DNA from these clones, as well as from parental unselected cells was prepared from about 10^6 cells in each case using the QIAamp Blood Kit™ (Qiagen Inc., Chatsworth, CA) and

resuspended at 0.5 mg/ml in sterile H_2O. Quantitative PCR was performed in 50 µl aliquots using 0.2 µg DNA, 50 pmol each of forward and reverse primers amplifying a 270 base pair region of exon 1 and intron A of the dihydrofolate reductase gene (see below), 50 mM KCL, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 250 mM dNTPs, 0.5 µl Tac polymerasse (Qiagen, Inc., Chatsworth, CA), 10 µl Q buffer™ (Qiagen, Inc., Chatsworth, CA) and 1 pmol radioactively end-labeled reverse primer (labeled with γ-^{32}P-dATP). Amplification conditions were as follows: 1 cycle: 94° C, 1'30"; 25 cycles: 94°(1 min)-57°(1 min)-70°(2'30"); 1 cycle:94°(1 min)-57°(1 min)-70°(7'). Control reactions with known amounts of DNA in two-fold dilutions were performed to insure that the extent of reaction was directly proportional to the amount of template. Following amplification, 10 µl aliquots were electrophoresed on a 1% agarose gel. The gel was vacuum-dried for two hours onto filter paper and the PCR-amplified 270 base pair band was quantitated using an Ambis4000 Radioanalytic Imaging system (Ambis, Inc., San Diego, CA).
Primer sequences for the dihydrofolate reductase gene were as follows: (forward primer) 5'-GGTTCGCTAAACTGCATCGTCGC-3' and (reverse primer) 5'-CAGAAATCAGCAACTGGGCCTCC-3'.

Apoptosis assay. Apoptosis was assayed using the Cell Death Detection ELISA (Boehringer Mannheim, Indianopolis, IN). 2 x 10^5 cells were plated in 24-well plates and infected the next day (when the cells were about 80% confluent) with Ad-p53 or Ad-ßgal as described above. 48 hours post-infection, cells were collected and cytoplasmic fractions were prepared and assayed for the presence of oligonucleosomal fragments,

which are released from the nuclei of the cells undergoing apoptosis, by following the manufacturer's protocol.

RESULTS

Cell lines. We have previously provided evidence that the JNK/SAPK pathway is involved in the cellular response to cisplatin, and that inhibition of this pathway in T98G cells modified to express a nonphosphorylatable dominant negative mutant of c-Jun, termed dnJun, blocks repair of cisplatin-DNA adducts and markedly increases sensitivity to cisplatin and other types of DNA damage (14). In Table 1, we summarize the growth properties of T98G parental cells and clones modified to express dnJun or wild-type c-Jun (control). Growth retardation does not correlate with expression of dnJun, as dnJun I-10-10 proliferates about 20% faster than parental cells or c-Jun-modified cells, and dnJun I-10-6 proliferates about 20% slower. This indicates that basal AP-1 activity associated with cell growth is not affected by the dnJun or cJun modification. Furthermore, dn Jun-expressing cells and parental and c-Jun-expressing have similar plating efficiencies. Therefore, expression of dn Jun in T98G glioblastoma inhibits their ability to repair DNA damage, but it is not growth suppressive in itself.

Amplification frequency of the dihydrofolate reductase (DHFR) gene. Because certain types of DNA repair defects are associated with increased genomic instability (20,21) and tumorigenesis (22,23), we examined genome instability in the T98G clones described above. We examined the frequency of gene amplification, one measure of genome instability, which has been observed to correlate with increased tumorigenicity (24-26). We studied amplification of the dihydrofolate reductase gene in the presence of

methotrexate concentrations of 5 times the LD50 and 9 times the LD50, concentrations at which gene amplification of dihydrofolate reductase is the predominant mechanism of resistance (27,28). Table 2 compares the frequency of methotrexate resistant colonies appearing during one month of continuous culture of the various cell lines in the presence of methotrexate. T98GdnJun I-10-10 produces methotrexare resistant colonies at about 20 times the frequency of the parental T98G cells, and T98GdnJun I-10-6 produces methotrexate resistant colonies at about 80 times the frequency of parental T98G cells. In order to verify gene amplification, several clones were picked at random from each selection and expanded. Genomic DNA was prepared and subjected to quantitative PCR analysis using ^{32}P-labeled primers amplifying a 270 base fragment of the dihydrofolate reductase gene including part of exon 1 and intron A. PCR products were analyzed by agarose gel electrophoresis and quantitated by radioanalytic imaging. The levels of the PCR amplification product were used to calculate the relative gene dosage level of the DHFR gene and are indicated in Table 2. T98GdnJun cells have from 2 to 4 times the gene dosage of the DHFR gene relative to parental T98G cells and T98GcJun cells

Suppression of T98G cell clones by p53. Because increased sensitivity to p53 expression has been observed to correlate with increased DNA damage (29), we tested whether the sensitivity of T98GdnJun cells rendered them more sensitive to wild-type p53 expression. T98G cells lack wild-type p53 function as a result of a methionine to isoleucine replacement in p53 at codon 237 (30), and we have confirmed this by sequencing the RT-PCR product of total cellular RNA from T98G cells (18). Restoration of wild-type p53 in T98G cells through gene transfer results in G1 arrest (30) or

apoptosis (18). Furthermore, agents that promote DNA damage enhance p53-mediated apoptosis (18). Figure 1 compares the growth inhibition of Ad-p53-transduced cells relative to Ad-ßgal-transduced cells 6 days post-infection. The results represent the average of two experiments performed on separate occasions, with each experiment being performed in triplicate. The infection efficiency under our conditions was about 50%, low enough to cause incomplete suppression of parental T98G cells and control cells modified with c-Jun. T98GdnJun I-10-10 and I-10-6 display considerably more growth suppression by p53 under these conditions.

Induction of apoptosis in the presence of p53. In order to determine whether the p53-mediated growth inhibition of T98GdnJun cells could be accounted for by the induction of apoptosis, we assayed the cytoplasmic fractions of Ad-p53 or Ad-ßgal-infected cells, 48 hours post-infection, for the presence of oligonucleosomal fragments. These fragments are released from the nuclei of cells during the early phase apoptosis, and can be detected by an ELISA assay using anti-histone antibodies and anti-DNA peroxidase antibodies (18). We assayed for apoptosis 48 hours following exposure to p53-adenovirus or ß-gal adenovirus, as maximal transgene expression in Ad-ß-gal-infected cells occurs between 48 and 72 hours post-infection (R.Gjerset and S. Lebedeva, unpublished). Figure 2A shows the results of the ELISA assay on the various T98G cell clones. Low levels of oligonucleosomal fragment release similar to levels observed in uninfected cells were observed in Ad-ß-gal-infected cells. Treatment of parental T98G cells and control cJun cells with Ad-p53 (100 pfu/cell, 3 hours) resulted in virtually no induction of apoptosis under our conditions, consistent with growth assays showing no

apoptosis (18). Furthermore, agents that promote DNA damage enhance p53-mediated apoptosis (18). Figure 1 compares the growth inhibition of Ad-p53-transduced cells relative to Ad-ßgal-transduced cells 6 days post-infection. The results represent the average of two experiments performed on separate occasions, with each experiment being performed in triplicate. The infection efficiency under our conditions was about 50%, low enough to cause incomplete suppression of parental T98G cells and control cells modified with c-Jun. T98GdnJun I-10-10 and I-10-6 display considerably more growth suppression by p53 under these conditions.

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suppression of overall growth following treatment of these cell lines with Ad-p53. However, significantly higher levels of apoptosis were observed in dnJun clones I-10-10 and I-10-6. These results are consistent with the marked cytopathic effects observed by 72 hours post-infection in Ad-p53-treated cultures of T98G I-10-10 and I-10-6 (Figure 2B). In contrast, Ad-p53-treated cultures of T98G cells and control c-Jun cells show less cytopathic effects. Taken together these results show that p53-mediated apoptosis is enhanced in dnJun-expressing cells and suggest that the Jun kinase pathway modulates sensitivity to p53, possibly through its effect on DNA repair and genome stability.

DISCUSSION

In this study we have examined the effects of a dominant-negative inhibitor of c-Jun phosphorylation on cell growth and genome stability in T98G glioblastoma cells. We have also examined the combined effects of this inhibitor and p53. Cells modified with dnJun grow with similar doubling times and have similar plating efficiencies as parental T98G cells or cJun-modified cells. However, we observe a 20 to 80 fold higher frequency of gene amplification, one measure of genome instability, and an increased sensitivity to p53-mediated growth suppression and apoptosis in dnJun-modified cells. Therefore dnJun alone had little effect on the growth properties of T98G cells but manifested a negative effect on growth in the presence of p53.

These results demonstrate that the Jun kinase pathway plays a role in the maintenance of genome stability, consistent with our earlier finding linking this pathway to DNA repair (14). The results provide evidence within a series of clones derived from a single cell line, that sensitivity to p53 expression correlates with increased genome instability and with decreased DNA repair. Our observations are consistent with other examples where DNA repair defects are seen to underlie a genome instability phenotype (20,21). The observations are also consistent with a recent study showing that within a given cell line expressing a fixed level of p53, the degree of DNA damage correlates with the apoptotic response. (29).

Our results can be understood in light of a growing body of evidence supporting a role for p53 in modulating apoptosis in response to DNA damage (see review, ref. 31), and in proportion to the extent of damage (29). P53 is a DNA damage recognition protein known to bind to a variety of types of DNA damage, including single stranded ends (2), and insertion-deletion loops (3). These types of damage, which could serve as triggers for p53-mediated apoptosis are likely to be generated in tumor cells by the mechanisms that promote spontaneous gene rearrangements, deletions, and amplifications. As such, the success or failure of DNA repair may be critical in determining how such a cell responds to expression of p53. By inhibiting repair of strand breaks, dnJun, may promote genome instability on the one hand, and enhance sensitivity to p53 on the other hand.

As depicted in the scheme in Figure 3, we hypothesize that induction of Jun kinase and loss of p53 represent independent mechanisms by which tumor cells undergoing progression accommodate increased levels of genomic instability and insure survival while sustaining potentially lethal genome destabilizing events. By promoting DNA repair, the Jun kinase pathway may limit damage to levels compatible with survival. Loss of p53 would further enhance survival by down-regulating the apoptotic response to unrepaired damage.

These observations have clinical implications with regard to the use of p53 to suppress the growth of tumors. As tumor progress, particularly if they have been exposed to DNA damaging therapies, cellular stress responses and DNA repair mechanisms may be

upregulated. Indeed, the Jun kinase pathway has been reported to play a role in tumorigenic progression of rat embryo fibroblasts (17) and in human tumor cell lines (32). In cells where cellular stress responses and DNA repair pathways have been upregulated in response to increasing levels of genome instability or in response to external DNA damaging treatments, the response to ectopic expression of p53 may be attenuated. Optimizing the benefits of p53-based therapies may therefore require a concurrent down-regulation of cellular stress pathways. Studies on the combined therapeutic benefits of p53 along with down-regulation of the Jun kinase pathway are currently in progress.

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Table 1. *Culture characteristics of T98G clones*

clone	relative doubling time[a]	plating efficiency[b]
T98G	1	47% ± 3%
c-Jun.	1.0 ± 0.14	52% ± 17%
dnJun I-10-10	0.83 ± 0.10	42% ± 13%
dnJun I-10-6	1.2 ± 0.04	45% ± 2%

a) Average of 2 experiments on different occasions, each in triplicate.
b) Average of 3 experiments on different occasions, each in triplicate.

Table 2. *Frequency of methotrexate(MTX)-resistant colonies in T98G cells and in c-Jun and dn-Jun-modified cells.*

Cell were plated at a density of 10^5 cells per 10-cm culture dish and allowed to 4 weeks in the presence of methotrexate at the indicated concentrations. Plates were then stained with 70% methylene blue in methanol and colonies were counted. For analysis of DHFR gene amplification, several colonies were picked at random prior to staining, expanded and cellular DNA was prepared and subjected to PCR analysis

Clone	MTX dose[a]	frequency of colonies per 10^5 cells (average of 3 experiments)	DHFR gene dosage relative to parental
T98G parental	5 x LD50 9 x LD50	2.0 0.3	1
c-Jun	5 x LD50 9 x LD50	2 0	n/t
dnJun I-10-10	5 x LD50 9 x LD50	40 10	3-4
dnJun I-10-6	5 x LD50 9 x LD50	174 16	2

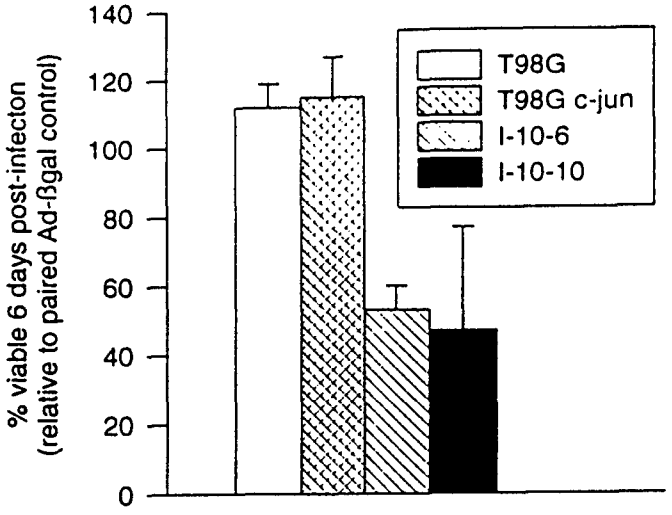
a) LD50 = 0.1 μM (T98G, c-Jun) or 0.04 μM (dnJun I-10-10, dnJun I-10-6).

Figure Legends

Figure 1. 6 day viability assay of T98G subclones following treatment with Ad-p53, 100 pfu/cell for 3 hours. Viability of each Ad-p53-treated cultures is represented as a percentage of the same culture treated under identical conditions with Ad-ßgal.

Figure 2. (A) ELISA apoptotis assay of cytoplasmic nucleosomes in untreated cells, or in cells 48 hours after being treated with 100 pfu per cell of Ad-ßgal or Ad-p53 for 3 hours. (B) Light microscopy (40x) of untreated cells (top row), or cells 72 hours following treatment with Ad-ßgal (middle row) or Ad-p53 (bottom row). Columns are as follows: (Column A) T98G parental cells, (Column B) c-Jun-modified cells, (Column C) dnJun I-10-6, (Column D) dnJun I-10-10.

Figure 3. Model explaining how dnJun increases the amount of p53 "substrate" (DNA damage) in genomically unstable tumor cells and thus collaborates with dnJun to promote p53-mediated apoptosis.



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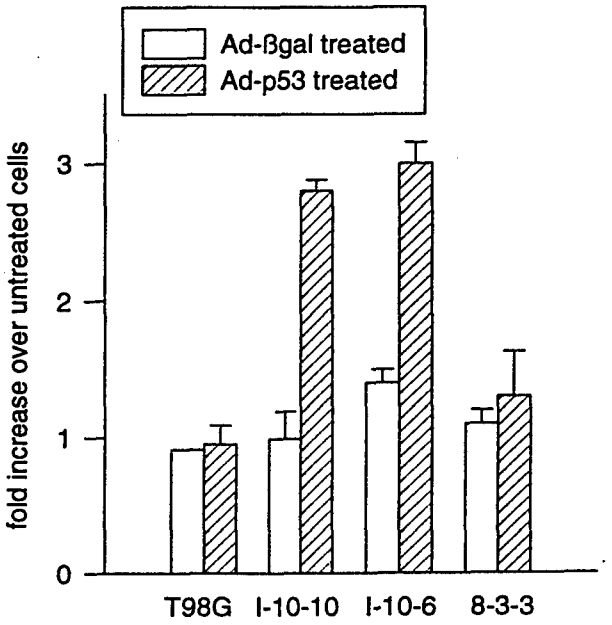

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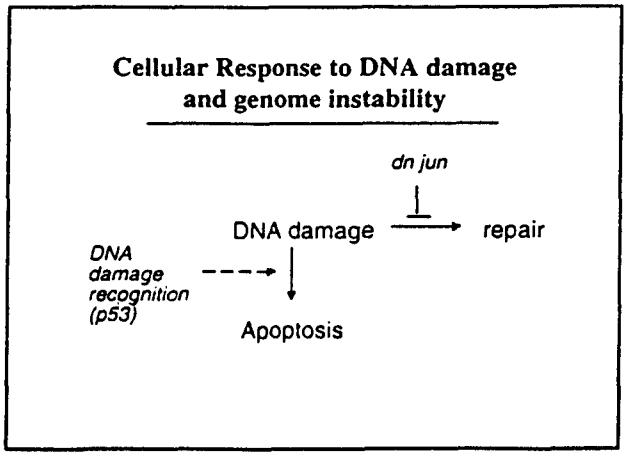

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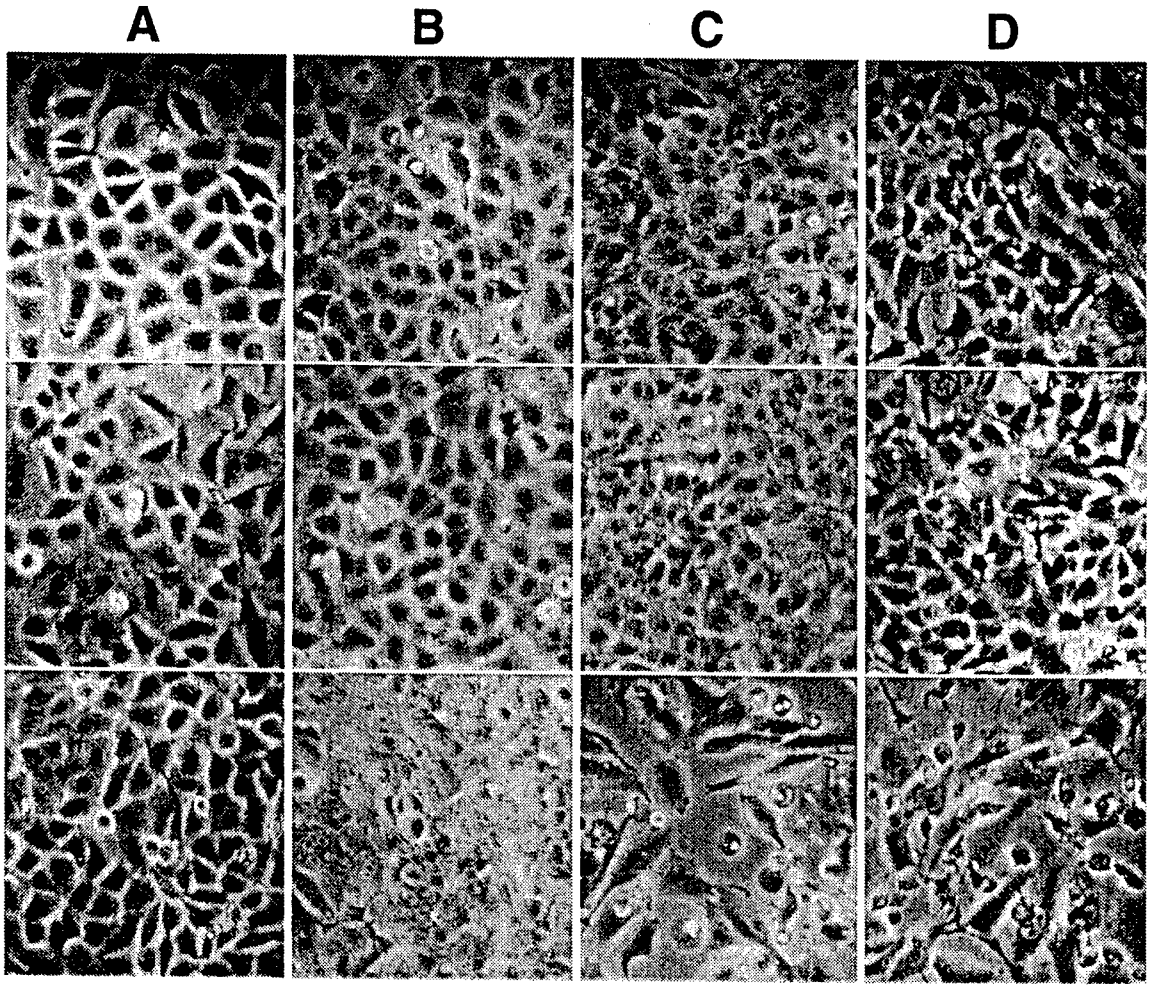

Figure 2B

DEPARTMENT OF THE ARMY
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504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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